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Experimental Hyperleptinemia in Neonatal Rats Leads to Selective Leptin Responsiveness, Hypertension, and Altered Myocardial Function

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Abstract—The prevalence of obesity among pregnant women is increasing. Evidence from human cohort studies and experimental animals suggests that offspring cardiovascular and metabolic function is compromised through early life exposure to maternal obesity. Previously, we reported that juvenile offspring of obese rats develop sympathetically mediated hypertension associated with neonatal hyperleptinemia. We have now addressed the hypothesis that neonatal exposure to raised leptin in the immediate postnatal period plays a causal role. Pups from lean Sprague-Dawley rats were treated either with leptin (3 mg/kg IP) or with saline twice daily from postnatal day 9 to 15 to mimic the exaggerated postnatal leptin surge observed in offspring of obese dams. Cardiovascular function was assessed by radiotelemetry at 30 days, and 2 and 12 months. In juvenile (30 days) leptin-treated rats, hearts were heavier and night-time (active period) systolic blood pressure was raised (mm Hg; mean \pm SEM: male leptin-treated, 132 \pm 1 versus saline-treated, 119 \pm 1, n=6, P <0.05; female leptin-treated, 132 \pm 2 versus saline-treated, 119 \pm 1, n=6, P <0.01), and the pressor response to restraint stress and leptin challenge increased compared with saline-treated rats. Heart rate variability demonstrated an increased low:high frequency ratio in 30-day leptin-treated animals, indicative of heightened sympathetic efferent tone. Echocardiography showed altered left ventricular structure and systolic function in 30-day female leptin versus saline-treated rats. These disorders persisted to adulthood. In isolated hearts, contractile function was impaired at 5 months in male leptin-treated rats. Exogenously imposed hyperleptinemia in neonatal rats permanently influences blood pressure and cardiac structure and function. (*Hypertension*. 2013;62:627-633.) • [Online Data Supplement](#)

Key Words: developmental programming ■ hypertension ■ leptin ■ obesity ■ rats ■ sympathetic nervous system ■ ventricular dysfunction

Pregnant women who are obese or whose gestational weight gain is above recommended limits are at increased risk of obstetric complications,¹ but there may also be longer term consequences for the health of the child.² A high maternal pre-pregnancy body mass index and excessive gestational weight gain have been frequently independently associated with a heightened risk of offspring metabolic dysfunction and obesity³ and there is increasing evidence to suggest that cardiovascular function may also be compromised.⁴

Studies of maternal calorie-rich diets and obesity in rodents, sheep, and nonhuman primates have provided unequivocal evidence for persistent and adverse influences on the offspring.⁵ We and others have repeatedly observed cardiovascular dysfunction secondary to maternal obesity in animal models.⁶⁻⁸ Previously, we observed that juvenile offspring of obese rats have aberrant autonomic control of blood pressure (BP) resulting in hypertension, which occurs before the development of

increased adiposity and persists into adulthood,⁷ and that these animals demonstrate hyperphagia and leptin resistance associated with a functional, structural, and cell-signaling deficit in leptin-sensitive processes in the arcuate nucleus and paraventricular nucleus of the hypothalamus.⁹ The cardiovascular response to exogenous leptin, which is mediated through hypothalamic sympathetic efferent activity,^{10,11} was, conversely, enhanced.⁷ This differential response to leptin, sometimes termed selective leptin resistance, had been observed in rodents, but previously only in association with chronic obesity.¹² We also reported that the offspring of obese dams (OffOb) exhibit an exaggerated and prolonged physiological postnatal serum leptin surge,⁹ known to play a critical role in the development of the normal rodent hypothalamus.¹³ We hypothesized that neonatal exposure to supranormal leptin concentrations during this important window of hypothalamic plasticity may play a causal role in offspring cardiovascular

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dysfunction. We have, therefore, investigated the influence of exogenous leptin administration in neonatal rats on parameters of cardiovascular function and control measured in juvenile and adult life. Because the heart weight is increased in offspring of obese rats^{9,14} and mice¹⁵ and because leptin has been implicated in neonatal rodent cardiomyocyte proliferation and remodeling,^{16,17} we also studied the morphology of the heart, and cardiac function as assessed in vivo and ex vivo.

Methods

Animals

Standard principles of laboratory animal care were used and all procedures approved in accordance with the United Kingdom Government Home Office Animals (Specific Procedures) Act, 1986 (PPL70/7090) and King's College London Animal Ethical Review Process Committee.

Female Sprague-Dawley rats (Harlan Laboratories) were housed under controlled conditions (light exposure, 07:00–19:00 hours; 21±2°C; 40%–50% humidity) and during mating and breeding maintained on a breeding diet (RM3, Special Diet Services). Forty-eight hours after delivery, litter size was standardized to 8 pups (equal numbers, male and female) and the litters randomly assigned to leptin (L-Tx; n=12) or saline (S-Tx; n=12) treatment. Leptin (3 mg/kg; PeptoTech Ltd) or saline vehicle was administered intraperitoneally twice daily (10:00 hours, 16:00 hours) from postnatal day (PD) 9 until PD15. Offspring were weaned (day 21) onto standard chow and euthanized at 30 days, 2 and 12 months of age. Blood was collected by cardiac puncture. Inguinal white adipose tissue, the heart, and the liver were weighed and cryopreserved. A separate cohort of L-Tx and S-Tx litters were euthanized by decapitation at PD 2, 6, 7, 8, 9, 11, 14, and 20, 4 hours after the first leptin injection to obtain organ weights and serum for leptin analysis.

Leptin Challenge

Animals were challenged with leptin at 30 days (juvenile) and 2 months of age (adult) after an overnight fast as described.⁹ A single bolus dose of recombinant rat leptin (10 mg/kg, IP) or saline vehicle was administered at 10:00 hours immediately before food restoration. The feeding and weight responses to leptin were assessed by measuring the change in body weight and food intake during the 24-hour period after the injection.

Radiotelemetry

Radiotelemetry methods have been described previously.⁷ Briefly, juvenile rats were implanted with a mouse transmitter (PA-C10, Data Sciences International [DSI]) in the aortic arch.⁶ In adults, the DSI rat telemetry probe (PA-C40) was placed in the descending abdominal aorta.⁷ Heart rate variability (HRV) was analyzed from the telemetry record. Cardiovascular response to restraint stress was recorded for 20 minutes with 120-minute recovery. Responses to a combination of intraperitoneal terazosin (10 mg/kg) and propranolol (10 mg/kg), or to leptin (10 mg/kg) were recorded.⁷ Spontaneous locomotor activity (counts of lateral movements in the cage) was monitored and analyzed using DataScience Dataquest software and expressed in arbitrary units.

Echocardiography

Left ventricular (LV) systolic function and chamber dimension were determined in vivo in 30-day and 12-month-old rats. M-mode tracings were recorded from the short-axis view at the high papillary muscle level using a high-resolution Vevo 770 echocardiography system (RMV707B, VisualSonics, Toronto, ON, Canada; see the online-only Data Supplement).

Isolated Perfused Heart Preparation

As LV function, assessed using electrocardiography, is load- and heart rate-dependent, we assessed LV function and remodeling ex

vivo at 30 days and 5 months of age, using isolated Langendorff heart preparations (see online-only Data Supplement).

Cardiomyocyte Morphology

Midventricular sections (7 µm) of paraformaldehyde fixed hearts were stained with laminin for determination of cardiomyocyte number and mean cardiomyocyte cross-sectional area. Sections were also stained with hematoxylin and eosin and Masson trichrome to estimate fibrosis (see online-only Data Supplement).

Analyses

Leptin and Insulin

The serum leptin and insulin concentrations were measured by ELISA (Crystal Chem Inc, Downers Grove, IL).

Renal Tissue Norepinephrine Content

The left kidney was homogenized in HCl (0.01 mol/L) with EDTA (1 mmol/L) and sodium metabisulfite (4 mmol/L) at day 30. After centrifugation (8000g; 30 minutes), the supernatant norepinephrine (NE) concentration was measured by ELISA (ALPCO Diagnostics, Salem, NH). NE content was expressed as nanogram per gram of renal tissue.

Data and Statistical Analysis

Data are presented as mean±SEM and analyzed using Student *t* test and 1-way ANOVA when appropriate. Cardiovascular analysis was performed by 1-way ANOVA for repeated measurements followed by the Tukey post hoc test. Where no significant differences between male and female data were observed using 2-way ANOVA, data were combined. Analysis of myocyte cross-sectional area (each animal given equal importance) was performed using linear regression with 100 measurements per animal, 4 sections per animal. Statistical significance was accepted as *P*<0.05.

Results

Organ Weight and Fat Mass

Neonatal leptin injection at PD9 to PD15 increased the serum leptin concentration ≈5 fold compared with saline treatment, when measured 4 hours postintraperitoneal injection (Figure S1A in the online-only Data Supplement). L-Tx pups demonstrated an increase in heart weight at PD9, PD11, and PD20 compared with S-Tx (Figure S1C) with no change in body weight (Figure S1B). L-Tx hearts were also heavier than S-Tx at day 30, and body and organ weights remained unchanged (Table S1). At 2 and 12 months, the heart weight in male and female L-Tx, as well as the inguinal fat mass, was increased relative to S-Tx (Table S2).

Leptin Challenge

Thirty-day-old L-Tx rats showed impaired anorectic responses to the leptin challenge compared with S-Tx, as demonstrated by an absence of a reduction in food intake or body weight over a 24-hour period (Figure S2A). At this time there was no increase in endogenous serum leptin or insulin concentration (Figure S3A and S3B). Leptin resistance was also present at 2 months of age in L-Tx (Figure S2B), at which time endogenous serum leptin and insulin concentrations were significantly raised compared with S-Tx (Figure S3A and S3B).

BP and Autonomic Function

At 30 days, L-Tx females had a higher mean arterial pressure (MAP) and night-time heart rate (HR; active phase)

versus S-Tx (Figure 1B). L-Tx males showed increased night-time HR versus S-Tx (Figure 1A). Systolic BP was increased in L-Tx male (night-time) and female rats versus S-Tx (Figure S4A and S4B). Diastolic BP was similar in L-Tx and S-Tx (Figure S4A and S4B). Male L-Tx demonstrated reduced locomotor activity versus S-Tx ($P<0.05$, Figure S4A).

At 2 months, there were no sex differences in BP, HR, or locomotor activity. L-Tx demonstrated increased day and night-time MAP ($P<0.05$ and $P<0.001$, respectively), whereas night-time HR was markedly reduced ($P<0.001$) versus S-Tx (Figure 1C). Night-time systolic BP ($P<0.05$) and day and night-time diastolic BP ($P<0.001$) were higher in L-Tx versus S-Tx (Figure S4C). There was no difference in spontaneous locomotor activity between groups (Figure S4C).

Spectral analysis of basal HRV showed an increased ratio of low-to-high frequency domains in 30-day-old L-Tx versus S-Tx, indicative of a dominant increase in sympathetic activity. Time domains of HRV, SD of normal to normal intervals and root mean square of successive differences, were similar in juvenile L-Tx and S-Tx. At 2 months of age, L-Tx (sexes combined) showed decreased SD of normal to normal intervals, high frequency, and increased low

frequency and the ratio of low-to-high frequency versus S-Tx rats (Table S3).

Cardiovascular Reactivity to Stress and Renal NE

Thirty-day-old L-Tx females exhibited a higher MAP ($P<0.01$) and HR ($P<0.05$) response to 20 minutes of restraint versus S-Tx (Figure S5B). After 120 minutes, the MAP and HR remained elevated in both male and female L-Tx versus S-Tx in which cardiovascular responses had returned to baseline (Figure S5A and S5B). Renal NE content (at baseline, no restraint) was increased 2-fold in L-Tx versus S-Tx (Figure S5C). Sympathetic inhibition by combined administration of the $\alpha 1$ -adrenergic receptor antagonist, terazosin, and the $\beta 1/\beta 2$ -adrenergic receptor antagonist, propranolol, normalized MAP and led to a greater fall in basal MAP in L-Tx rats (sexes combined) versus S-Tx (Figure 2A and 2B).

BP Response to Leptin Challenge

At 30 days of age, intraperitoneal administration of leptin increased MAP in both L-Tx and S-Tx rats (sexes combined) within 2 hours (Figure 3A). The increase in MAP (Δ MAP) was twice as high in L-Tx versus S-Tx rats 6 hours post challenge (Figure 3B). MAP returned to baseline in all the animals by 8 hours (Figure 3A).

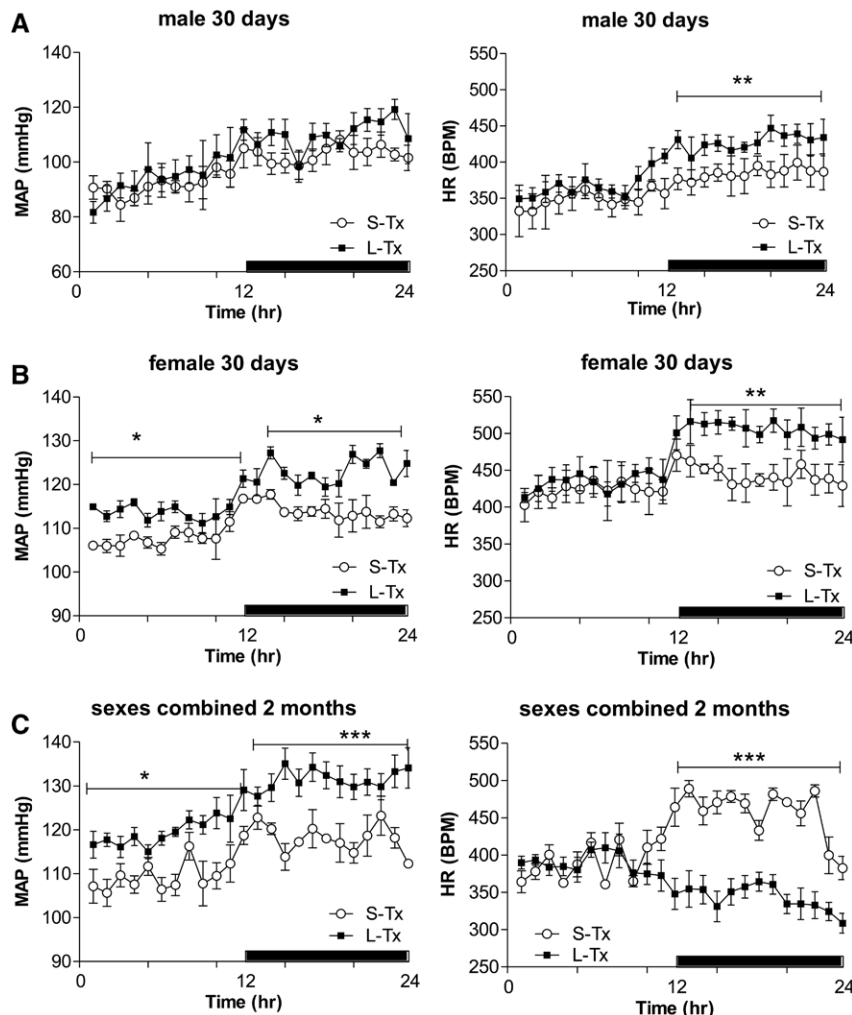


Figure 1. Mean arterial pressure (MAP) and heart rate (HR) in 30-day-old saline-treated (S-Tx; open circles) or leptin-treated (L-Tx; closed circles) male (A) and female (B) rats and 2-month-old male and female rats (C). *** $P<0.001$; ** $P<0.01$; and * $P<0.05$ vs saline (repeated-ANOVA; $n=4$). BPM indicates beats per minute.

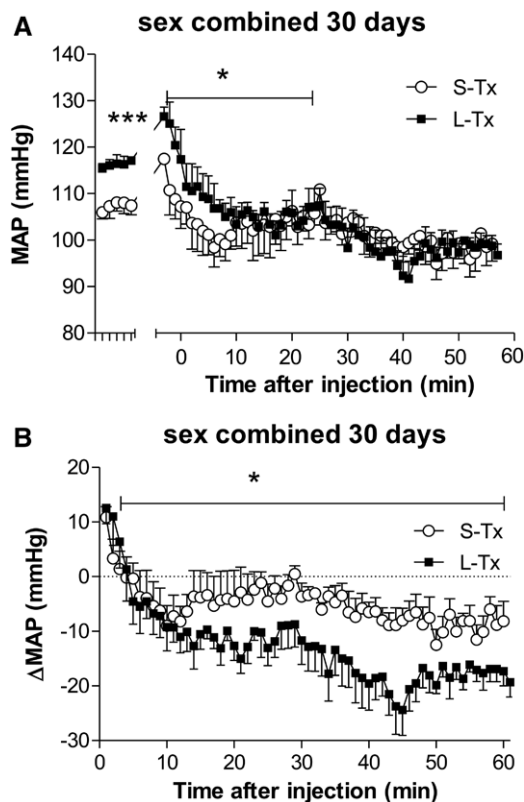


Figure 2. Mean arterial pressure (MAP) response (A) and Δ MAP (B) to α 1-adrenergic receptor antagonist, terazosin (10 mg/kg, IP), plus a β 1/ β 2-adrenergic receptor antagonist, propranolol (10 mg/kg, IP) in 30-day-old saline-treated (S-Tx; open circles) or leptin-treated (L-Tx; closed circles) male and female rats combined. *** P <0.001 vs saline-treated (prechallenge); * P <0.05 vs saline-treated (15 min, A; 60 min, B; repeated-ANOVA; n =5–6).

Cardiac Structure and Function

Thirty-day-old female L-Tx showed increased LV internal diameter at systole, increased LV volume at systole, and decreased intraventricular septal thickness at diastole versus S-Tx (Figure S6A). This was associated with a reduced ejection fraction and fractional shortening in female L-Tx versus S-Tx (Figure S6A; Table S4).

At 12 months, female L-Tx showed markedly increased LV mass, LV internal diameter at systole, and LV posterior wall at diastole (Figure S6B; Table S5). Female L-Tx also demonstrated impaired cardiac function with reduced ejection fraction and fractional shortening (Table S5). Male L-Tx showed increased LV mass and LV internal diameter at diastole (Table S5). No differences in function were observed between groups in male rats.

Isolated Hearts

Thirty-day-old male and female S-Tx and L-Tx showed preserved Frank-Starling responses to increased LV pressure (Figure S7A and S7B). At 5 months, Frank-Starling responses were similar in S-Tx and L-Tx, whereas L-Tx male hearts showed impaired LV systolic function at a given level of preload (160–260 μ L), whereas diastolic stiffness remained unaffected (Figure S7A). No differences in cardiac systolic or diastolic function were observed in 5-month-old L-Tx females (Figure S7B).

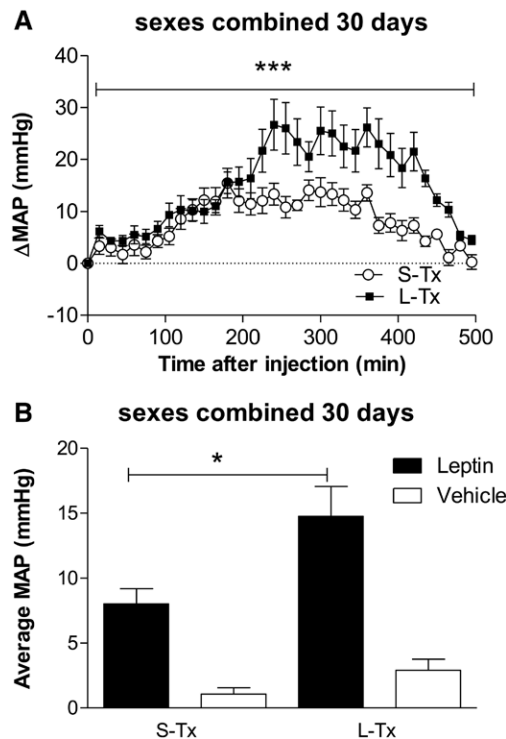


Figure 3. Mean arterial pressure (MAP) response to leptin (10 mg/kg, IP) in 30-day-old saline-treated (S-Tx; open circles) or leptin-treated (L-Tx; closed circles) male and female rats combined (A). Calculated average response to leptin and vehicle over 6 h post challenge (B). *** P <0.001 vs saline-treated (repeated-ANOVA); * P <0.05 vs saline-treated (t test; n =6–8).

Isoprenaline (10 nmol/L) stimulation resulted in a positive inotropic and chronotropic response in S-Tx and L-Tx hearts, which was independent of age (Figure S8). This chronotropic response was blunted in male L-Tx versus S-Tx hearts at 30 days, although no differences in inotropic responses were observed (Figure S8A). No differences in inotropic or chronotropic response were observed in females at any age (Figure S8B).

Cardiomyocyte Morphology

Left ventricle myocyte cross-sectional area showed similar Gaussian distribution between the groups (Figure 4A). There was no significant mean difference in cross-sectional area between groups. Cardiomyocyte number per unit area was, however, increased in L-Tx versus S-Tx rats (Figure 4B). Histological examination showed no evidence of fibrosis in the perivascular or intramural vascular structures in L-Tx hearts at 30 days of age (Figure S9).

Discussion

We have previously reported that maternal diet-induced obesity in rats is associated with juvenile hypertension, hyperphagia, and leptin resistance in the offspring.⁷ We implicated central pathways in the hypothalamus and proposed a causal role for the exaggerated and prolonged leptin surge observed in the neonatal offspring during the second postnatal week of life.^{7,9} Leptin plays a physiological neurotrophic role in hypothalamic development during this critical period.¹⁸ We now demonstrate that exogenous leptin given to rat pups from lean

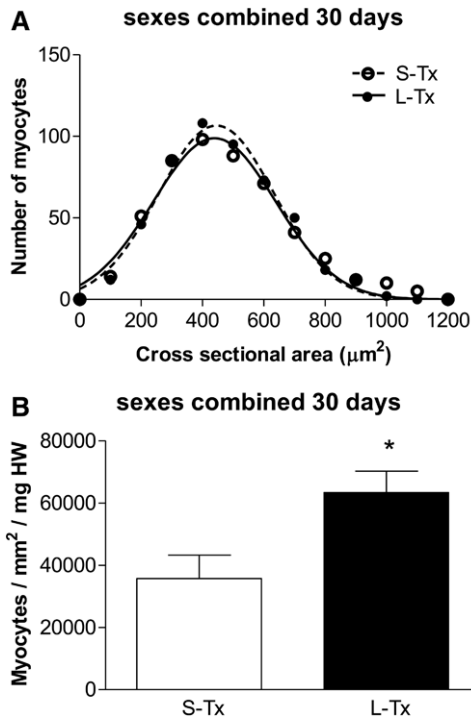


Figure 4. Myocyte cross-sectional area (CSA; **A**) and number (**B**) from left ventricular (LV) myocardial sections of 30-day-old saline-treated (S-Tx) and leptin-treated (L-Tx) rats. A similar Gaussian distribution for LV myocyte CSA was observed between the groups (100 measurements per animal, 4 sections per animal). Increased cardiomyocyte number (myocyte/ mm^2 /mg heart weight (HW) was shown in L-Tx vs S-Tx. Values are measured per unit area from 6 fields per section, 4 sections per animal. * $P < 0.05$ vs saline (t test).

dams during the same period leads to a similar phenotype and conclude that this hormone may play a key mechanistic role in the relationship between maternal obesity and cardiovascular and metabolic dysfunction of the offspring.

The serum concentration of leptin achieved in the pups by injection was higher (≈ 2 -fold), but of the same order of magnitude, as that at the peak of the neonatal leptin surge in the OffOb.⁹ Similar to the OffOb rats, juvenile L-Tx rats showed a loss of the inhibitory effects of leptin on food intake and body weight, at a time at which endogenous serum leptin was not raised, and this leptin resistance continued into adult life. This is consistent with previous reports showing that exogenous leptin treatment during early postnatal life in rodents leads to a permanent loss of the anorexic response to leptin and increased diet-induced weight gain in adulthood.^{18–20}

In common with juvenile OffOb rats,⁷ L-Tx rats demonstrated early onset hypertension, increased stress reactivity, renal NE content, and changes to the HRV and the heart rate relationship with BP, indicative of an increase in central sympathetic activity. Further proof of sympathetically mediated hypertension was provided by normalization of the BP by pharmacological adrenoceptor blockade. Despite showing no increase in fat mass or hyperleptinemia at this age, these animals also demonstrated an increased pressor response to leptin, another characteristic of the OffOb rat.⁷ This too implicates increased sympathetic efferent activity, as leptin plays an important cardiovascular regulatory role

in the central nervous system.¹¹ Infusion of leptin or leptin overexpression in adult mice increases renal sympathetic nerve activity and BP,^{10,12} whereas monogenic leptin deficiency, in both humans and animals, leads to obesity, but protects against obesity-related hypertension.^{21,22} Several of these reports implicate a central role for the hypothalamus, also the site of leptin induced appetite regulation. Taken together the data suggest that the cardiovascular and appetite-regulatory effects of neonatal leptin treatment are attributable to divergent effects on discrete signaling pathways resulting in selective leptin responsiveness in different regions of the hypothalamus, a hypothesis also proposed from leptin challenge studies in adult animals.^{11,12} Also, because there is evidence of leptin receptors in the nucleus of the solitary tract which affect sympathetic efferent activation, a role for this cannot be discounted.²³ Previously reported in adult animals and humans which are obese and hyperleptinemic,^{12,24} the concept that selective leptin responsiveness can be induced by early exposure to exogenous neonatal leptin is a novel observation. Overall, these observations lend strong support to our hypothesis that the exaggerated postnatal leptin surge in OffOb rats plays an essential role in the adulthood cardiovascular and metabolic profile previously reported.^{6,15}

To our knowledge only 2 previous studies have assessed the influence of neonatal leptin on subsequent cardiovascular function. When leptin was administered to mice in the neonatal period (80 ng/g IP PND4–14), Erkonen et al²⁵ measuring BP with a tail cuff, reported reversal of hypertension in 4-month-old mice which had been induced by neonatal growth restriction (achieved through large-litter rearing), but BP in normal weight pups was unaffected. Trevenzoli et al²⁶ have recently described increased BP, also by the tail cuff method, in adult 150-day-old rats treated with leptin in the neonatal period (PD1–10), achieving concentrations comparable with the present study, but this could have arisen from the associated increase in body weight in adulthood. The novelty of the present study lies in the development of sympathetically mediated hypertension in juveniles after neonatal L-Tx, before the development of hyperleptinemia and increased body weight, suggesting a direct influence of early leptin exposure on the developing pathways of BP control. Importantly, the use of radiotelemetry avoided stress activation of sympathetic tone, which can be induced by the tail cuff technique.

The BP profile in L-Tx and OffOb rats was not entirely similar. Whereas at 30 and 90 days of age, both male and female OffOb rats had abnormal MAP,⁷ only L-Tx females demonstrated a higher MAP. Leptin may, therefore, not be alone in playing an influential role. Fetal hyperinsulinemia also affects hypothalamic neurodevelopment,²⁷ and the glucose intolerance observed in obese rodent dams is likely to stimulate fetal insulin secretion.^{6,14}

Persistent increase in heart weight has been observed in OffOb rodents.^{6,14} The alterations in cardiac function in juvenile L-Tx rats as assessed by echocardiography were similar to those we observed in a preliminary study in adult OffOb mice.²⁸ Cardiac dilatation observed together with impaired contractility (ejection fraction, fractional shortening) may reflect a second decompensatory phase which may be a prelude to myocardial failure in this model.²⁹

As L-Tx rats were hypertensive at each age studied, the cardiac abnormalities may occur simply as a result of an increase in BP and hemodynamic load or altered neurohumoral signaling. Alternatively, leptin treatment in early life may have a persistent influence on cardiac structure; previous reports have shown that the addition of leptin to rat neonatal myocyte culture leads to hypertrophy¹⁷ or hyperplasia.¹⁶ Because the majority of rat cardiomyocytes undergo terminal maturation in early postnatal life,³⁰ we hypothesized that postnatal hyperleptinemia could induce cardiac dysfunction in the juvenile animal through either of these processes. There was no evidence of myocyte hypertrophy and we conclude that increased cardiac mass is a consequence of increased myocyte number, as calculated. Expansion of the extracellular matrix may play a role, but there was no histological evidence of fibrosis.

Sympathetic activation may stimulate heart growth.³¹ In the older animals, impaired contractility may also be influenced by hyperleptinemia.³²

In male L-Tx rats, cardiac function gradually deteriorated with age, possibly related to secondary effects of increased adiposity, hyperleptinemia, and hyperinsulinemia. In contrast, in female L-Tx rats, age-related effects were not obvious, providing an example of sexual dimorphism, frequently observed in studies of developmental programming.³³ There were also differences between female systolic function as assessed in vivo and ex vivo, implicating a role for neural or hormonal influences in vivo.

Care must be taken in extrapolating from animal models to the human condition. Rodents provide a convenient experimental model, with a relatively fast life course, but they are altricial species born at an immature stage of development compared with humans. Considering the critical postnatal window in the current study, the equivalent period of developmental plasticity in the human hypothalamus is most likely the third trimester of pregnancy, but may extend into postnatal life, as demonstrated in nonhuman primates.³⁴ The metabolic profiles in the adult OffOb rats show similarities to that found in nonhuman primates and in sheep, supporting translation to precocial species.⁵ The fetuses of obese pregnant women are exposed to hyperleptinemia as measured by cord blood leptin³⁵ and in mothers with type 1 diabetes mellitus, cord blood leptin correlates with childhood body mass index aged 7 years³⁶ and, although seldom measured, BP has been reported to be raised in adult offspring of obese women.⁴

Perspectives

We propose that early life exposure to hyperleptinemia may directly predispose to early onset hypertension, hyperphagia, and cardiac dysfunction. Mechanistically this may involve the hypothalamus. We have previously shown impaired development of the agouti-related protein pathway in the hypothalamus of juvenile OffOb rats.⁹ Agouti-related protein is the endogenous antagonist of the melanocortin-4 receptor; therefore, a reduced antagonism may increase melanocortin-4 receptor signaling at sites relevant to BP regulation and consequently to hypertension,³⁷ a concept open to exploration with specific pharmacological agents. This study also opens avenues of exploration in animal models and in humans to address the role of early life leptin exposure in early onset

essential hypertension. Most importantly, ongoing mother–child cohort in obese pregnant women should address associations between maternal obesity and offspring cardiovascular function in young children, and through to adulthood.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Neonatal exposure to hyperleptinemia in rodents leads to early onset hypertension, hyperphagia, and cardiac dysfunction, before the development of hyperleptinemia and obesity.
- Hyperleptinemia in the immediate postnatal period leads to an adult phenotype similar to that described in offspring of obese rodents.
- Increased leptin exposure secondary to maternal obesity may predispose to early hypertension and heart failure.

What Is Relevant?

- High leptin concentrations, secondary to maternal obesity might constitute a cardiovascular risk for the offspring.

- This study suggests 1 potential mechanism for the development of early essential hypertension.

Summary

Neonatal hyperleptinemia plays an important role in the developmental origin of hypertension, secondary to maternal obesity in rodents. Exposure to leptin during critical periods of development also influences cardiac structure and function in a way which may predispose to heart failure in adulthood.

Experimental Hyperleptinemia in Neonatal Rats Leads to Selective Leptin Responsiveness, Hypertension, and Altered Myocardial Function

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ONLINE SUPPLEMENT

**Experimental Hyperleptinemia in Neonatal Rats Leads to Selective Leptin Responsivness,
Hypertension and Altered Myocardial Function**

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Expanded Materials and Methods

Telemetry and heart rate variability

Baseline systolic blood pressure (SBP), mean arterial pressure (MAP), diastolic blood pressure (DBP), heart rate (HR) and physical activity were measured in S-Tx and L-Tx rats at 30 days and 2 months of age using radiotelemetry.¹ Briefly, rats were anesthetized with 2 % isoflourane in O₂ at 2 l per minute with pre- and post-operative analgesia (buprenorphine, 0.1 mg kg⁻¹S.C.). In juvenile rats (23 days), a small transmitter designed for the mouse (DSI PhysioTel® PA-C10) was implanted in the aortic arch via the left carotid artery.² In adult rats (50 days), the standard rat telemetry blood pressure probe (DSI PhysioTel® PA-C40; Data Sciences International) was placed in the descending abdominal aorta as previously described.³ Following one week of recovery, rats housed in individual cages were placed above the telemetric receiver. Cardiovascular variables and physical activity were routinely monitored in a quiet room by scheduled sampling for 10 seconds every 5 minutes with A.R.T. 10 software (Dataquest 3.01, DSI, St Pauls, MN). Data reported are for a single 24 hour period.

Heart rate variability (HRV) was analysed from a 300 seconds continuous telemetric BP record (HR derived from pressure waves) using spectral analysis. Data sets recorded in a sinus rhythm with sampling frequency 500 Hz were used. Time and frequency domain of HRV analysis were performed using HRV module of Chart 5.0 analysing software (ADInstruments, Colorado Springs, CO). Ectopics and visible short artefacts were manually excluded or replaced by intervals linearly interpolated from the nearest normal interval to avoid discontinuity in the record. Spectral powers of BP signals were analysed with the LabVIEW 7.1 (National Instruments, MA). Spectral segments were set to 0.20-0.60 Hz for low frequency (LF), which is an index of both sympathetic and parasympathetic activity, and 0.6-2.5 Hz for high frequency (HF), which is an index of parasympathetic nervous activity.⁴ The power and frequency of every spectral component are presented in absolute units.

Power (in m²) was estimated as the area under the spectrum within these frequency ranges. Beat-by-beat time series of blood pressure waveform were generated, and series of pulse intervals (PI) were obtained by measuring the intervals between consecutive BP waves. In the time-domain, the following indices were obtained: R-R (mean R-R interval), SDNN (standard deviation of normalised R-R intervals) and RMSSD (root mean square of successive differences of R-R intervals).

Cardiovascular responses to restrain stress and adrenergic receptor blockade

Telemetered animals were subjected to stress using a humane Perspex whole body restraining cylinder. The size of the cylinder was selected according to body size. Cardiovascular parameters were recorded in animals 30 minutes before the stress, 20 minutes during the stress and for 120 minutes after return to the home cage. Experiments were performed between 0900 and 1000 hours. Scheduled sampling was implemented for 10 seconds every minute (Dataquest LabPRO Aquisition System version 3.01, DSI, St Pauls, MN).

Two days after the stress test a sub-group of animals received a combination of the $\alpha 1$ adrenergic receptor antagonist, terazosin (10mg/kg, i.p), plus the $\beta 1/\beta 2$ adrenergic receptor antagonist, propranolol (10mg/kg, i.p, Sigma –Aldrich Ltd, Poole, UK). Cardiovascular parameters were recorded for 30 minutes before the injection (baseline) and then for 10

seconds every minute for 2 hours after the injection. The receptor blockade achieved with this protocol has been previously demonstrated.⁵

Cardiovascular response to leptin

Randomly selected age-matched littermates were given a single bolus dose of IP leptin (10 mg/kg BW; PeproTech) or vehicle. The pressor effects were assessed before and 6 hours after injection, using scheduled sampling (10 seconds every 15 minutes).

Echocardiography

Prior to the imaging studies, the rat was anesthetized (2-3% isoflurane mixed with 0.5 L/min 100% O₂) in the induction chamber. The investigator was blind as to whether the animals had been treated with saline or leptin in the neonatal period. After loss of the righting reflex, animals were placed on a warming platform and an appropriately sized nose cone was placed over the animal's nose. Anaesthesia was maintained using 0.5-1% isoflurane. Temperature was monitored with a rectal thermometer and maintained ~37 °C. Echocardiograms were performed using a Vevo 770 High Resolution Imaging System (VisualSonics, Toronto, ON, Canada) with a RMV-707B transducer running at 30MHz (in juvenile rats) and a RMV701B transducer running at 28MHz for the adult rats. High-resolution, 2- dimensional B-mode and M-mode images at the level of the papillary muscles were acquired. The following parameters were assessed in end-systolic and end-diastolic situation: thickness of the interventricular septum (IVS), left ventricle posterior wall thickness (LVPW), left ventricle inner diameter (LVID) and left ventricle volume (LVVol). Ejection fraction (EF), fractional shortening (FS), stroke volume (SV) and cardiac output (CO) were calculated with Vevo Software (VisualSonics). Measurements were made of the interventricular septum in diastole, left ventricular internal dimension in diastole, left ventricular internal dimension in systole and left ventricular posterior wall in diastole. These measurements were then used to calculate the left ventricular fractional shortening (FS), ejection fraction (EF), stroke volume (SV) and cardiac output (CO). Measurements were made in accordance with the American Society for Echocardiography Guidelines.⁶

Isolated Perfused Heart Preparation

At the age of 30 days or 5 months, hearts were excised and studied in a Langendorff isolated heart preparations. Age-matched rats were anesthetised with sodium pentobarbital (200 mg/kg and 200 IU/kg, respectively, ip). Hearts were rapidly excised, placed in cold Krebs-Henseleit buffer (KHB) and cannulated via the aorta. Hearts were then perfused with oxygenated (95% O₂ and 5% CO₂) KHB at 37.0°C (pH 7.4) in a retrograde Langendorff perfusion system at a constant pressure equivalent to 72±1 mmHg using a peristaltic pump and a feedback-system controlled by an STH Pump Controller (AD Instruments, Aus). The KHB contained (in mM) 118.5 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 25.0 NaHCO₃, 1.2 MgCl₂, 1.4 CaCl₂ and 11.1 glucose. Measures of cardiac contractile function, such as left ventricular developed pressure (LVDP) and HR, were performed with a liquid-filled balloon inserted into the left ventricle. After the baseline was established (~25 minutes), the pressure-volume Frank-Starling response was assessed by increasing the balloon volume in 20µl increments every minute, using a pre-calibrated syringe, until the new steady state was reached. The assessment of the Frank-Starling response was completed within 15 minutes and the balloon was deflated to the baseline level (left ventricle developed pressure, LVEDP, between 4-8 mmHg) for 5 minutes. Hearts were then perfused with KHB with isoprenaline (10nM) for further 15 minutes.

1 **Immunohistochemistry**

2 Immediately after euthanasia at postnatal day 30, the heart was snap-frozen in an isopentane-
3 dry ice slurry and stored at -80°C. The cardiac ventricles were cut (at 7µm) with a cryostat in
4 horizontal sections at the midventricular short axis, mounted on poly-lysine-coated glass
5 slides and fixed with ice-cold 4% PFA for 15 minutes. After removal of the fixative, sections
6 were permeabilized by the addition of 0.1% Triton-X-20 in PBS (Sigma-Aldrich, UK).
7 Sections were then incubated overnight at room temperature with a rabbit anti-laminin
8 (1:300, DakoCytomation, Glostrup, DE). Slides were then washed 3 times for 5 minutes with
9 0.1% Triton-X-20 in PBS. A secondary antibody, fluorescein (FITC)-conjugated goat anti-
10 rabbit IgG (1:400, Invitrogen, Carlsbad, CA) was applied for 2 hours then washed 3 times.
11 Slides were cover-slipped and examined by a fluorescence light microscope.

12 **Image Acquisition**

13 Laminin stained sections were used to quantify cardiomyocyte cross-sectional area (CSA)
14 and number. Digital images for laminin (green) staining were captured at x40 objective from
15 two non-overlapping fields (left ventricle) from each section (4 adjacent sections per heart),
16 using the AxioCam CCD digital camera (Carl Zeiss, Oberkochen, Germany) mounted to an
17 inverted epifluorescence microscope (AxioVert 135; Carl Zeiss). The CSA of individual
18 cardiomyocytes was quantified as previously described.⁷ For cell size, CSA was outlined
19 from 100 measurements per animal, 4 sections per animal, by two independent investigators
20 blind to the animal group. For cell number, myocytes were counted from 6 randomly selected
21 fields (x40 objective) per section, 4 sections per animals and expressed as number of
22 myocytes per unit area (1mm²).

23 **Test for Fibrosis**

24 Hearts were isolated and fixed with 4% formaldehyde, dehydrated and embedded in paraffin.
25 Global heart architecture was determined from 5µm deparaffinized sections stained with
26 hematoxylin and eosin (H&E). The possible presence of fibrosis was checked with Masson's
27 Trichrome staining.

28
29

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Tables and Figures

Table S1. Body weight, inguinal WAT, and heart -weight in 30 day- old male and female rats treated with saline (S-Tx) or leptin (L-Tx) in neonatal period.

Weight	Male		Female	
	S-Tx	L-Tx	S-Tx	L-Tx
Body weight (g)	63.5±2.6	65.2±1.2	62.0±1.1	63.5±1.9
Inguinal WAT (g)	0.18±0.03	0.20±0.03	0.15±0.03	0.14±0.01
Heart weight (g)	0.35±0.02	0.41±0.01*	0.35±0.01	0.42±0.01*
Liver weight (g)	2.53±0.13	2.58±0.10	2.33±0.10	2.60±0.32
Heart weight (g/kg BW)	5.53±0.17	6.34±0.28*	5.64±0.18	6.52±0.09*
Liver weight (g/kg BW)	40.2±2.9	39.1±1.7	38.7±2.0	42.3±2.6

Values given as mean± SEM * $P < 0.05$ versus saline treated using *t*-test, $n = 4-8$.

Table S2. Body weight, inguinal WAT, heart, and liver weight in 2 and 12 month old male and female rats treated with saline or leptin in neonatal period.

Weight	2 months				12 months			
	Male		Female		Male		Female	
	S-Tx	L-Tx	S-Tx	L-Tx	S-Tx	L-Tx	S-Tx	L-Tx
BW (g)	222.9±6.9	238.2±12.1	188.5±10.9	194.9±9.19	600.0±29.6	631.3±23.7	346.8±7.2	402.2±17.2†
Inguinal WAT (g)	1.32±0.15	2.03±0.35*	1.01±0.15	1.86±0.05†	5.31±0.41	9.25±1.21†	5.23±0.76	12.3±1.35‡
Heart weight (g)	0.87±0.02	1.12±0.09*	0.71±0.02	0.82±0.03*	1.61±0.03	1.77±0.03*	1.19±0.03	1.56±0.10†
Liver weight (g)	9.08±0.29	8.82±0.29	7.35±0.79	7.57±0.79	16.81±0.42	19.12±1.00	9.16±0.60	10.77±0.05

*P<0.05, †P<0.01, ‡P<0.001 versus saline treated using t-test, n=4-8. Abbreviation S-Tx saline treated, L-Tx leptin treated, BW body weight, WAT white adipose tissue.

Table S3. Heart rate variability in 30 day- and 2 month old male and female rats treated with saline or leptin in neonatal period.

Parameters	30 days				2 months	
	Male		Female		Male + Female	
	S-Tx	L-Tx	S-Tx	L-Tx	S-Tx	L-Tx
SDNN (ms)	7.9 ±1.3	4.8±0.8	3.4±0.4	2.7±0.5	8.1 ±0.3	3.2±0.2*
RMSSD (ms)	3.6±0.5	3.1±1.2	3.1±0.4	2.0±0.6	2.9±0.6	2.4±0.3
AverageHR (bpm)	372.3±9.1	370.1±7.7	342.0±9.9	342.0±15.1	400.1±10.2	406.2±4.1
RR (mean m²)	161.5±4.0	162.3±3.5	175.6±4.9	172.9±7.5	149.2±3.1	138.2±0.9
LF (ms²)	4.8±1.6	6.8±1.7	0.7±0.3	1.0±0.5	3.2±0.4	9.2±0.1*
HF (ms²)	9.5±4.2	8.5±4.2	3.6±0.9	2.3±1.2	6.5±1.2	3.2±0.3*
LF/HF	0.6±0.1	0.8±0.1*	0.2±0.1	0.5±0.1*	0.5±0.1	2.9±0.1‡

Values given as mean ± SEM *P<0.05, ‡P<0.001, versus saline treated using t-test, n=6-8 for leptin treated L-Tx versus saline treated S-Tx, using t-test, n=4-8. Time and frequency domains of the heart rate (HR) variability identify differences in the high frequency domain (HF; 0.60-2.5 Hz), corresponding to parasympathetic activity, and in the low frequency domain (LF, 0.20-0.60 Hz) corresponding to preliminary sympathetic activity. ⁴ Power (in m²) was estimated as the area under the spectrum within these frequency ranges. Beat-by beat time series of blood pressure waveform were generated, and series of pulse intervals (PI) were obtained by measuring the intervals between consecutive BP waves. In the time-domain, the following indices were obtained: R-R (mean RR interval), SDNN (standard deviation of normalised R-R intervals) and RMSSD (root mean square of successive differences of R-R intervals).

Table S4. Echocardiographic parameters measured in 30 day old male and female rats treated with saline or leptin in neonatal period.

Structure & Function	Male		Female	
	S-Tx	L-Tx	S-Tx	L-Tx
LVmass (mg)	282.8±10.0	281.6±12.0	284.1±13.9	271.6±10.5
LVVol;d (µl)	151.0±11.0	157.4±3.7	147.8±5.0	144.1±7.7
LVVol;s (µl)	43.3±7.02	38.8±2.3	28.8±2.3	34.8±3.1*
LVIDd (mm)	5.4±0.08	5.5±0.09	5.2±0.07	5.2±0.09
LVIDs (mm)	3.3±0.11	3.3±0.07	2.8±0.04	3.2±0.14*
LVPWd (mm)	1.3±0.03	1.2±0.07	1.3±0.03	1.2±0.04
LVPWs (mm)	1.9±0.13	2.0±0.10	2.0±0.08	1.8±0.11
SV (µl)	107.6±5.4	118.6±2.7	118.9±5.2	109.3±5.9
EF (%)	67.6±2.0	69.7±0.9	75.7±1.0	67.9±2.6*
FS (%)	38.2±1.6	39.8±0.7	45.0±1.0	38.5±2.0*
CO (ml/min)	43.7±2.7	46.5±1.8	52.6±2.9	45.8±2.8
HR (bpm)	406±9	395±9	438±13	420±11

Values are given as mean ± SEM *P<0.05 versus saline treated by t-test, n=4-8. Abbreviation S-Tx saline treated, L-Tx leptin treated. Left ventricular anterior wall at diastole (LVAW:d); Left ventricular anterior wall at systole (LVAW:s); Left ventricular internal dimension at diastole (LVID:d); Left ventricular internal dimension at systole (LVID:s); Left ventricular posterior wall at diastole (LVPW:d); Left ventricular posterior wall at systole (LVPW:s); From the PLAX view, the following measurements were obtained Intraventricular septum at diastole (IVS:d), mm; Intraventricular septum at systole (IVS:s), mm; Left ventricular internal dimension at diastole (LVID:d), mm; Left ventricular internal dimension at systole (LVID:s), mm; Left ventricular posterior wall at diastole (LVPW:d), mm; Left ventricular posterior wall at systole (LVPW:s), mm; Left ventricular internal volume at diastole (LVVol:d), ml; Left ventricular internal volume at systole (LVVol:s), ml; Stroke volume (SV), ml/min; Ejection Fraction (EF), %; Fractional Shortening (FS), %; Cardiac Output (CO).

Table S5. Echocardiographic parameters measured in 12 month old male and female rats treated with saline or leptin in neonatal period.

Structure & Function	Male		Female	
	S-Tx	L-Tx	S-Tx	L-Tx
LVmass (mg)	949.3±57.0	1217.6±45.1*	790.3±20.8	1005.3±72.5*
LVVol;d (µl)	316.1±45.9	378.3±59.6	345.8±6.6	392.7±26.9
LVVol;s (µl)	112.5±8.3	150.7±33.9	101.3±5.3	155.2±25.9*
LVIDd (mm)	7.8±0.22	8.9±0.05*	4.7±0.13	8.0±0.12
LVIDs (mm)	5.0±0.05	5.7±0.26	4.7±0.13	5.3±0.13*
LVPWd (mm)	2.0±0.04	2.0±0.18	1.8±0.04	2.2±0.15*
LVPWs (mm)	3.0±0.15	3.0±0.13	2.9±0.19	2.9±0.13
SV (µl)	203.7±37.8	227.6±28.2	244.5±3.4	237.6±15.2
EF (%)	63.5±3.2	61.1±3.8	70.8±1.1	61.1±4.8*
FS (%)	36.0±2.5	34.3±2.6	41.7±1.0	34.6±3.6*
CO (ml/min)	75.9±16.3	75.3±10.4	77.4±2.6	77.9±6.1
HR (bpm)	370.3±24	329±12	317±13	327±9

Values are given as mean ± SEM *P<0.05 versus saline treated by t-test, n=3-6. Abbreviation S-Tx saline treated, L-Tx leptin treated. Left ventricular anterior wall at diastole (LVAW:d); Left ventricular anterior wall at systole (LVAW:s); Left ventricular internal dimension at diastole (LVID:d); Left ventricular internal dimension at systole (LVID:s); Left ventricular posterior wall at diastole (LVPW:d); Left ventricular posterior wall at systole (LVPW:s); From the PLAX view, the following measurements were obtained Intraventricular septum at diastole (IVS:d), mm; Intraventricular septum at systole (IVS:s), mm; Left ventricular internal dimension at diastole (LVID:d), mm; Left ventricular internal dimension at systole (LVID:s), mm; Left ventricular posterior wall at diastole (LVPW:d), mm; Left ventricular posterior wall at systole (LVPW:s), mm; Left ventricular internal volume at diastole (LVVol:d), ml; Left ventricular internal volume at systole (LVVol:s), ml; Stroke volume (SV), ml/min; Ejection Fraction (EF), %; Fractional Shortening (FS), %; Cardiac Output (CO).

Figure S1

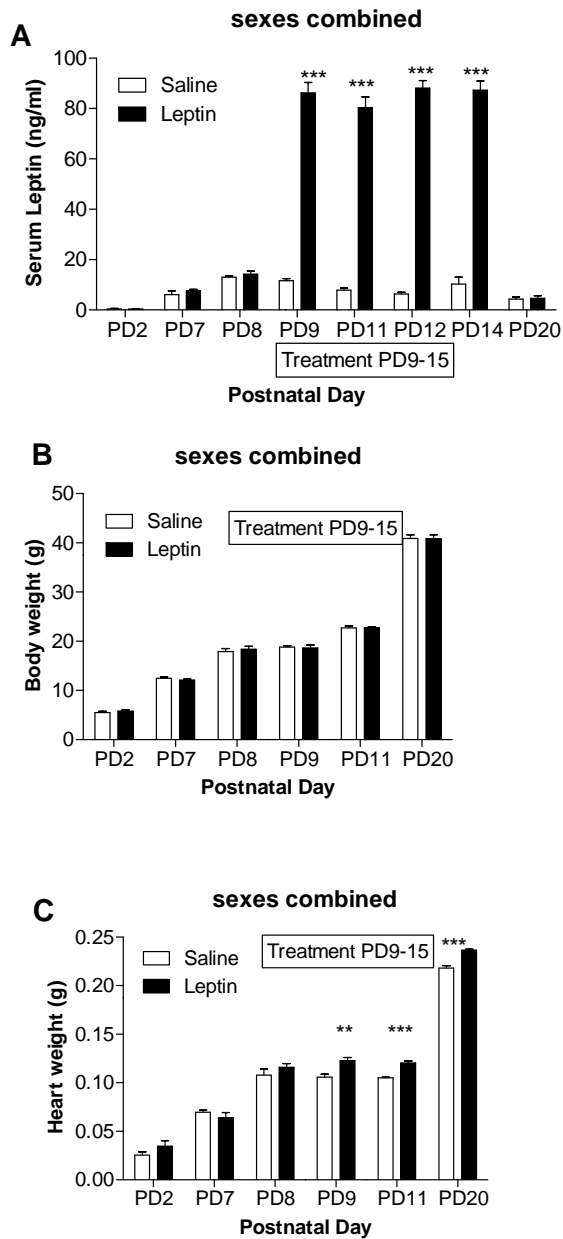


Figure S1. Neonatal serum leptin concentration (A), body weight (B) and heart weight (C) in leptin (3 mg/kg ip) and saline treated pups (male and female combined). *** $P < 0.001$, ** $P < 0.01$ versus saline using t-test, $n = 4-7$.

Figure S2

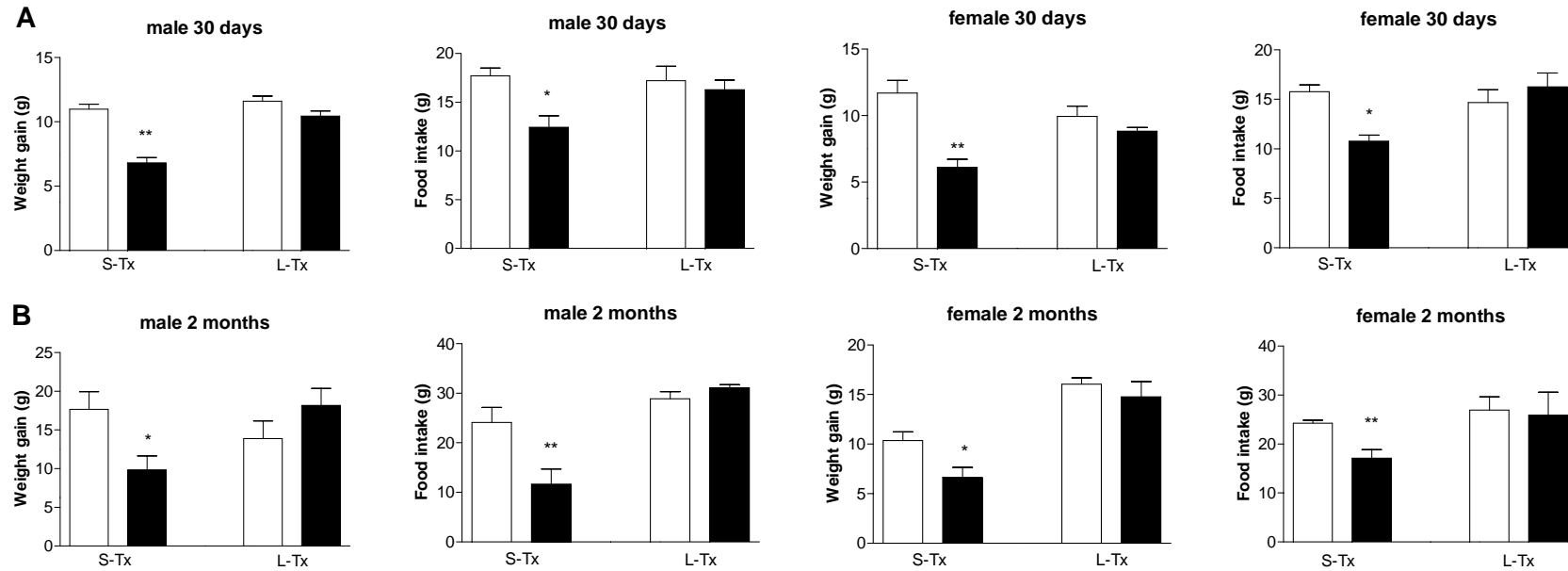


Figure S2. Weight gain and food intake recorded over 24 hours following a bolus leptin challenge (10 mg/kg i.p.) in saline-treated (S-Tx) or leptin-treated (L-Tx) male and female rats following an overnight fast at (A) 30 days and (B) 2 months of age. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus saline injection using t-test, $n=4-8$ Abbreviation S-Tx-saline treated; L-Tx-leptin treated

Figure S3

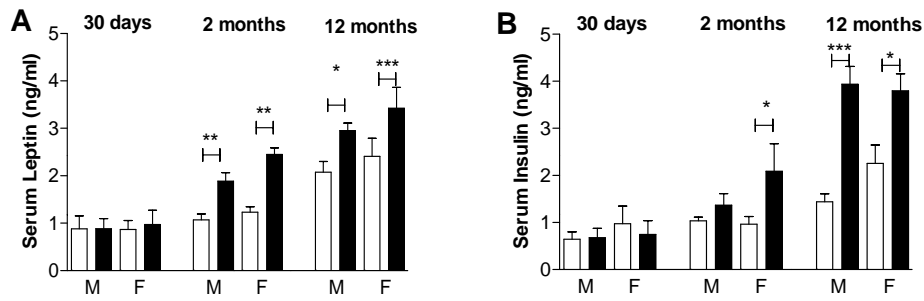


Figure S3. Serum leptin (A) and insulin (B) concentrations in saline-treated (open bars) or leptin-treated (closed bars) male and female rats at 30 days, 2 months and 12 months of age. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus saline using t-test, $n = 5-8$. Abbreviation M-male: F-female.

Figure S4

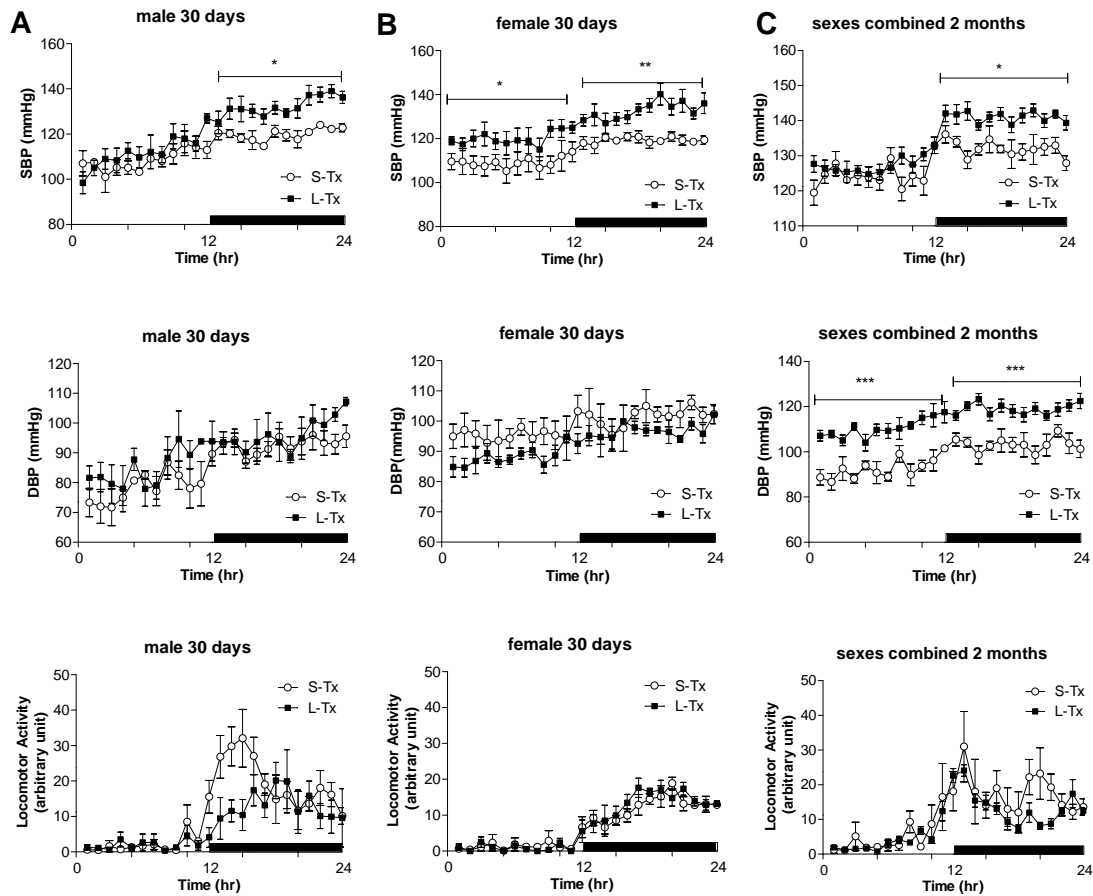


Figure S4. Systolic blood pressure (SBP), Diastolic blood pressure (DBP) and Spontaneous Locomotor Activity (arbitrary units) in 30 day old saline-treated (open circles) or leptin-treated (closed circles) male (A) and female (B) rats and 2 month old male and female rats combined (C). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus saline using repeated ANOVA, $n = 4-5$. Abbreviation S-Tx-saline treated; L-Tx-leptin treated.

Figure S5

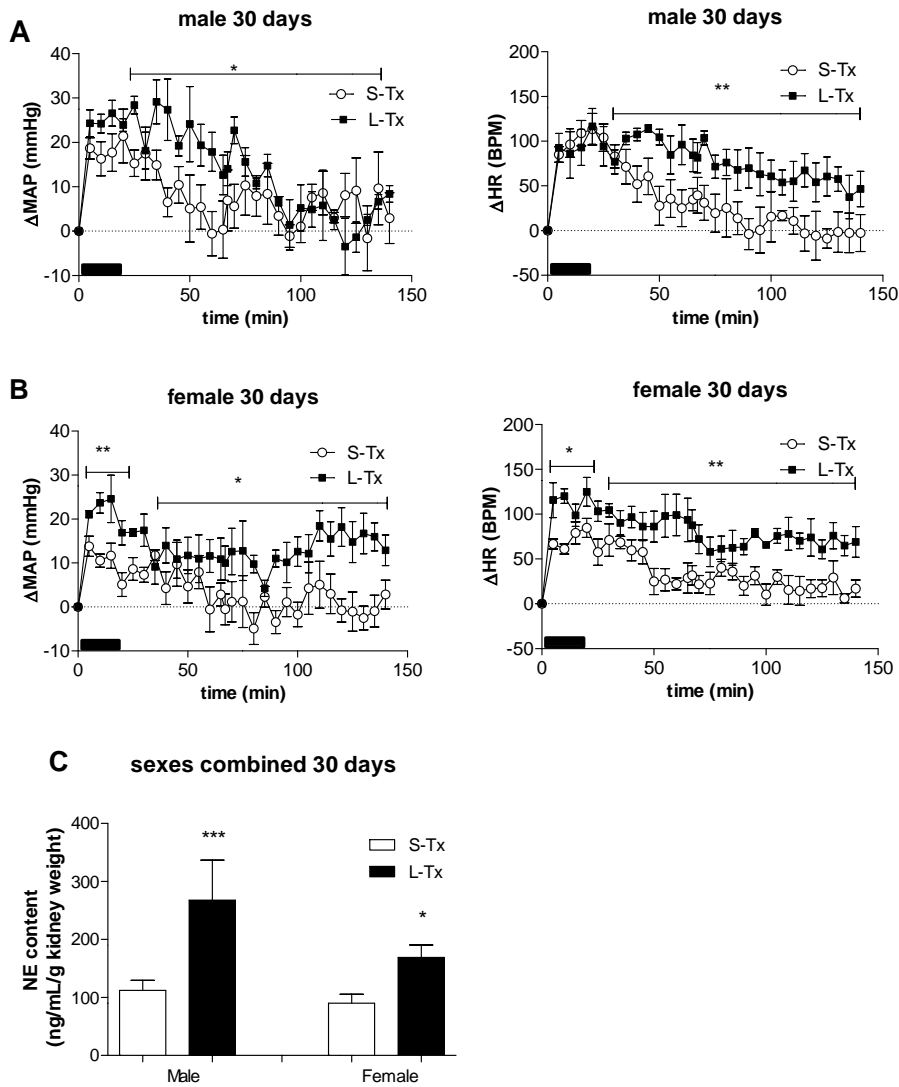


Figure S5. Mean arterial pressure (MAP) and heart rate (HR) response to restraint stress in 30 day old saline-treated (open circles) or leptin-treated (closed circles) male (A) and female (B) rats and renal NE content (C). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus saline using repeated-measures ANOVA and t-test, $n = 5-8$. Abbreviation S-Tx-saline treated; L-Tx-leptin treated.

Figure S6

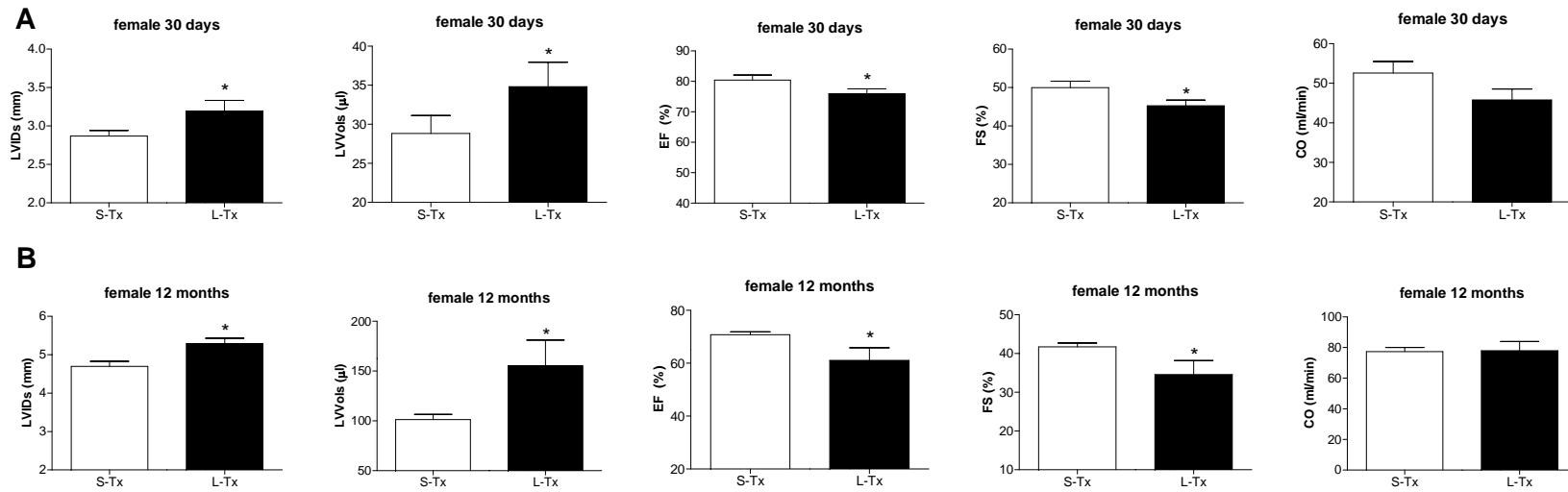


Figure S6. Cardiac structure and function from ultrasound analysis of saline-treated (open bars) and leptin-treated (closed bars) female rats at (A) 30 days and (B) 12 months of age. * $P < 0.05$ versus saline using t-test, $n = 4-6$. Abbreviation S-Tx-saline treated; L-Tx-leptin treated.

Figure S7

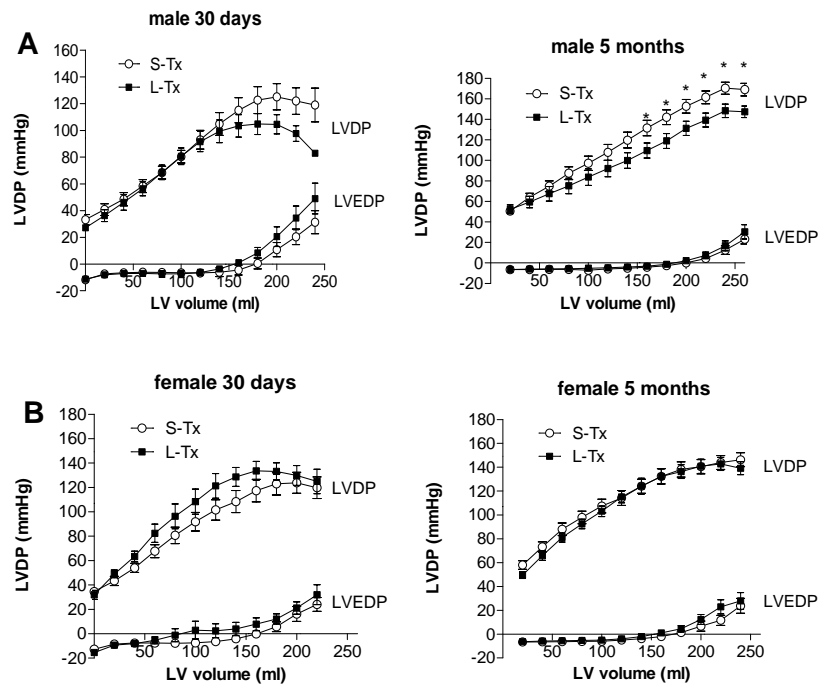


Figure S7. Frank-Starling response of isolated Langendorff perfused heart of saline-treated (open circles) or leptin-treated (closed circles) male (A) and female (B) rats at 30 days and 5 months of age. * $P < 0.05$ versus saline using repeated t-test, $n=6-10$. Abbreviation S-Tx-saline treated; L-Tx-leptin treated.

Figure S8

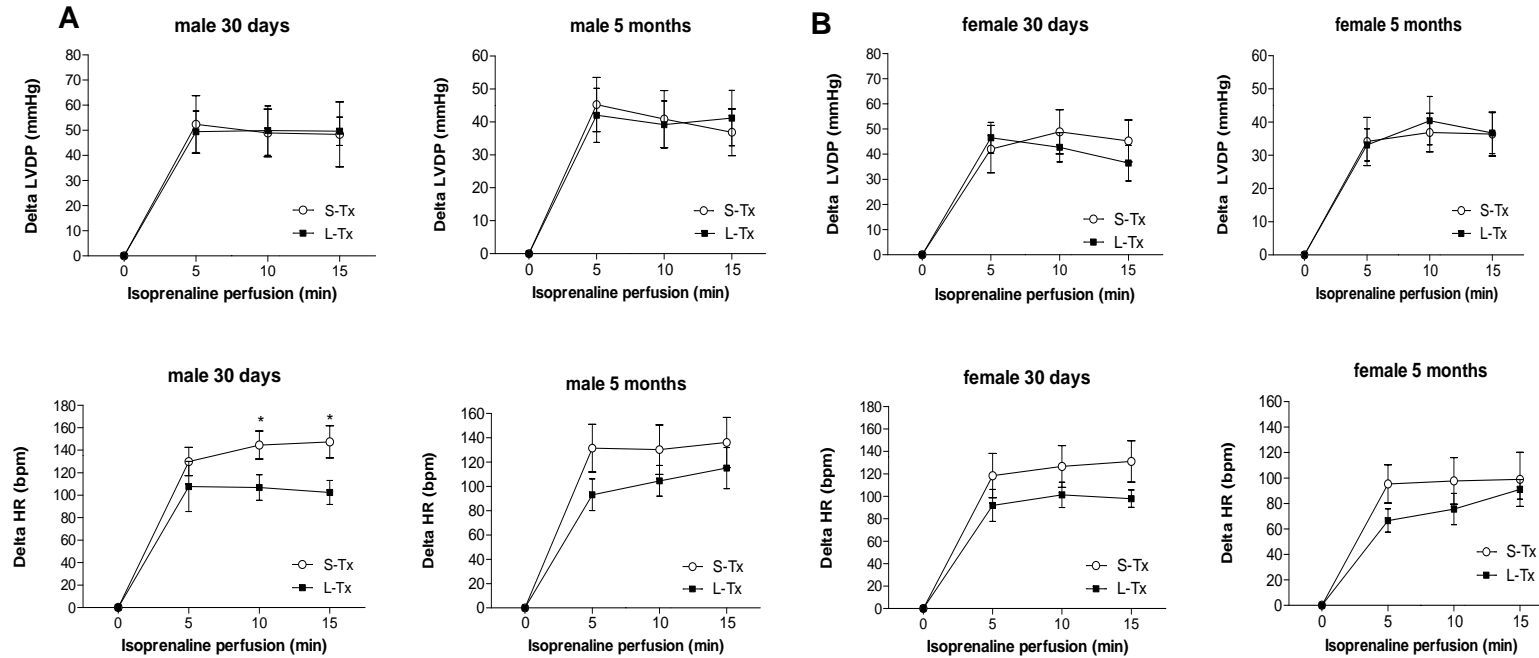


Figure S8. Frank-Starling response of isolated Langendorff perfused heart of saline-treated (open circles) or leptin treated (closed circles) male (A) and female (B) rats at 30 days and 5 months of age. * $P < 0.05$ versus saline using repeated t-test, $n = 6-10$. Abbreviation S-Tx-saline treated; L-Tx-leptin treated.

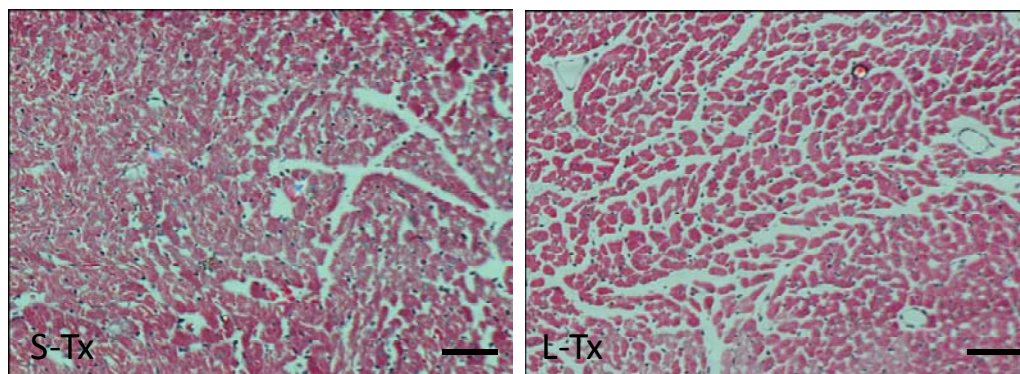


Figure S9. Representative images of H&E and Masson's Trichrome stained heart sections from 30 day old S-Tx and L-Tx rats. No fibrotic lesions were apparent. Scale bars: 100 μ m